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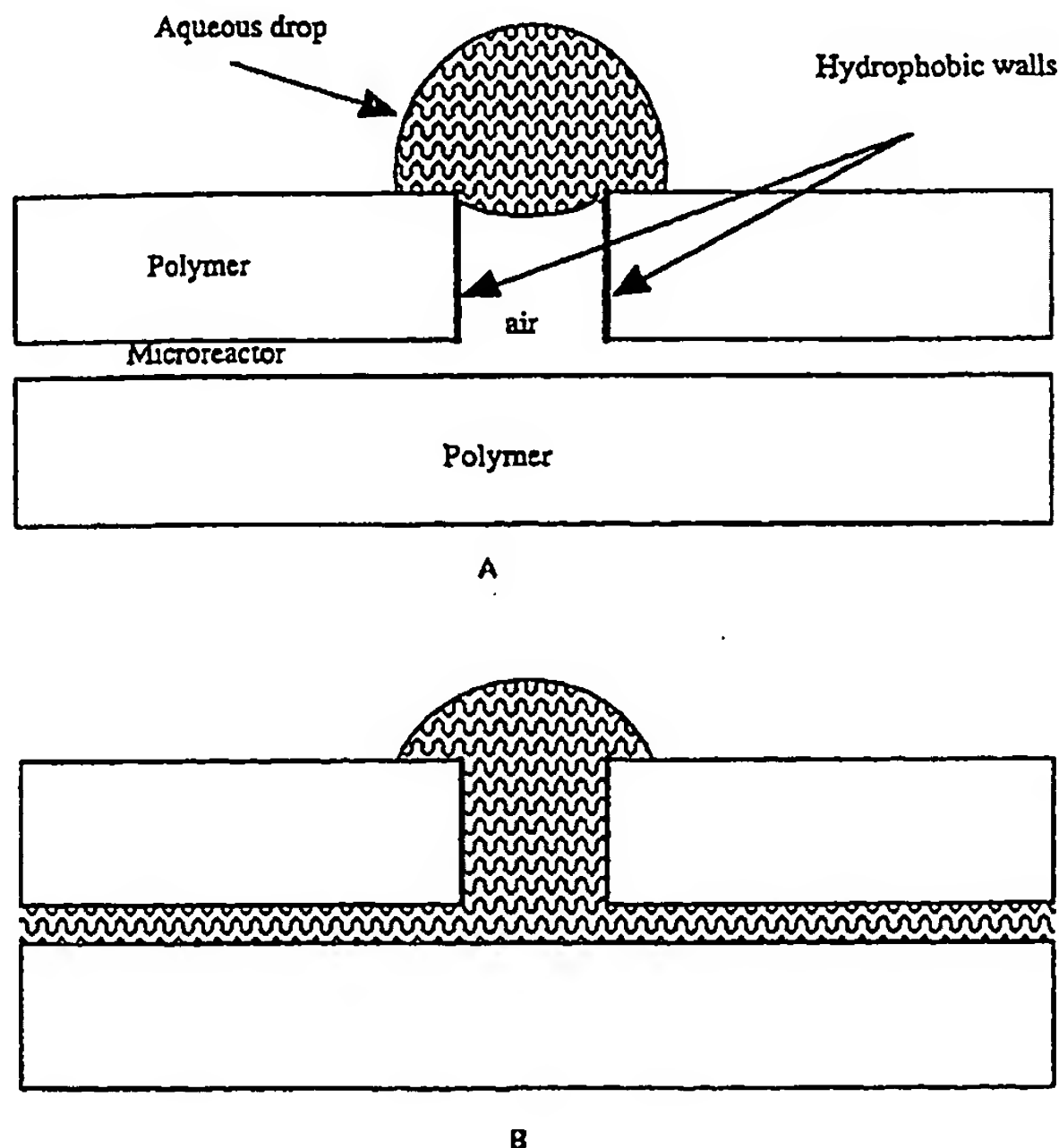
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(54) Title: MICROSCALE TOTAL ANALYSIS SYSTEM

(57) Abstract

The invention relates to an apparatus for performing chemical assays in an aqueous medium. The apparatus contains a reaction chamber(s) and a liquid in-flow channel connected to each chamber. The flow of liquid through the fluid in-flow channel to the reaction chamber is controlled by the presence of a hydrophobic inner surface on the walls of the in-flow channel. Under normal conditions fluid will not flow through the channel. However, application of an external force pushes the liquid through said channel into the reaction chamber. The invention is applicable to the monitoring of many different molecular interactions, in particular molecular recognition between an immobilised affinity partner and a species in solution, such as immunoglobulin/antigen interaction, DNA hybridisation, haptamer-protein interaction, drug and virus detection, high throughput screening of synthetic molecules and for determining the concentration and reaction kinetics of target species.



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MICROSCALE TOTAL ANALYSIS SYSTEM

This invention relates to apparatus for detecting the presence of a target species in an aqueous sample, and also to apparatus for determining the concentration and reaction kinetics of target species. The invention is applicable to the monitoring of many different molecular interactions, in particular molecular recognition between an immobilised affinity partner and a species in solution, such as immunoglobulin/antigen interaction, DNA hybridisation, haptamer-protein interaction, drug and virus detection and high throughput screening of synthetic molecules.

As many affinity complexations between two reaction partners are diffusion controlled, the time needed to reach reaction equilibrium is directly dependent on the mass transport of the molecule. The diffusion time of a molecule in a solution is proportional to the square of the path length; typically a small molecule needs less than one second to diffuse through 10 μm while it needs two hours to traverse one millimetre. In order to decrease the equilibrium time of the reaction, the chemical partners must therefore be placed as close as possible to each other; by reducing the reactor size to microdimensions, immobilising one partner on the surface of the reactor and filling the reactor with the second partner, the equilibrium time can be dramatically decreased.

The use of microreactors not only enhances the speed of affinity assays, but also facilitates the obtaining of information concerning reaction kinetics, which is important in the understanding of the thermodynamic stability of complexes. The affinity constant K_a is the ratio between the forward and reverse reaction rate constants k_1 and k_{-1} , which represent the association and the dissociation constants respectively. A strong complexation is characterised by a very fast association and a very slow dissociation, which in the particular case of sorbent affinity assays are adsorption and desorption from the

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surface of the microreactor. The understanding of these thermodynamic properties can be used for the study of cross-reactivity between several antigens or of non-specific adsorption of a matrix element during an affinity assay. By modulating the incubation time of the solution, the complexation of higher affinity partners is favoured and the non-specific adsorption is then reduced to a minimum. This fact may be an important factor in the decrease of the detection limit in immunosorbent assays due to the minimisation of the background signal. This method can also be applied to monitor the adsorption of antigens of different molecular weight.

As the diffusion coefficient of a molecule is proportional to its mass, the diffusion time of the molecule through the reaction chamber is different for small and large molecules. In the case of molecules of different molecular weight with the same epitope, (e.g. fibrine degradation products), the K_d may be the same for all molecules, whereas the diffusion coefficient is different for each of them. When a kinetics experiment is carried out, the smaller molecules will quantitatively reach the antibodies before the larger ones. The monitoring of the signal resulting from the affinity reaction as a function of time can thus deliver useful kinetics information, which can help in understanding the degradation process. These kinetics events can be followed by modulating the residence time of molecules in contact with their reaction partners, which can most readily be achieved by immobilising an antibody on the walls of a series of microreactors and by incubating different solutions of the analyte of interest for different periods of time.

In the past, analytical procedures of the type described above (such as enzyme-linked immunosorbent assays - ELISAs) have been performed using microtiter plates and have been relatively slow. In recent years, great efforts have been made to reduce the size of analytical devices to micrometer scale with the effect of reducing reaction times.

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These miniaturised systems have been termed "microscale total analysis systems" (μ -TAS)¹, and they have already been recognised as convenient means of manipulating and analysing small sample quantities ²⁻⁸. Most μ -TAS devices to date
5 have been produced by photolithography, wet chemical etching or thin film deposition on substrates such as glass, quartz and silicon ^{9, 10}. In order to decrease production costs, plastic substrates have also been micromachined using either silicone rubber casting ¹¹⁻¹⁴ injection moulding ¹⁵ embossing
10 ^{16, 17} or laser photoablation ¹⁸. These structures are planar devices with channels of micrometre size that are often sealed by thermal or anodic bonding to a glass cover. Interconnected channels may be fabricated easily, which makes possible the rapid separation and reactions in volumes
15 of few a picoliters. Other advantages of μ -TAS are the reduction of sample and reagent consumption and the increase of precision and reproducibility relative to bench scale apparatus ^{21, 22}.

Competitive immunoassays have also been performed on
20 micro-chips ²³⁻²⁵, but the micro-channels have only been used to electrophoretically separate free and bound forms of antigen or antibody. In these assays, antibody and labelled antigen are added in specific quantities to the sample to be analysed. The sample is then incubated with a
25 mixture of the labelled and native antigens that compete for a limited number of antibody binding sites. The micro-channel is then used to separate the free labelled antigen from the complex by capillary electrophoresis, and quantitation is performed by luminescence (fluorescence or
30 chemiluminescence) at the end of the separation channel. The amount of free labelled antigen measured is then related to the analyte concentration in the sample using a previously determined calibration curve. In this type of assay it is essential to avoid adsorption of a reaction partner on the
35 micro-channel walls.

Another type of immunoassay device has been developed for simultaneous analysis of multiple samples ²⁶. In this

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case, biotin-labelled antibodies are patterned onto an avidin-coated waveguide so as to form an array of six vertically oriented stripes of captured antibodies immobilised on the waveguide surface by avidin-biotin
5 bridges. Samples are then analysed using a sandwich immunoassay format by patterning another array of six horizontally oriented lines containing the corresponding fluorescent-labelled antigen at various concentrations. Fluorescent complexes on the surface of the waveguide are
10 then excited by a diode laser, and the fluorescence intensities of the 36 square dots is collected by a CCD camera. This immunosensor allows the analysis of multiple samples in parallel and simultaneous detection of more than one analyte per sample.

15 Numerous analytical methods utilise luminescence to detect an analyte of interest. Luminescence is the generic term referring to the emission of an electromagnetic radiation (UV, visible or IR) by an excited molecule that relaxes to its ground state, can be induced by
20 photoexcitation (photoluminescence) or by a chemical reaction (chemiluminescence and electrochemiluminescence). Chemifluorescence (CF) is another class of luminescent reactions which combines the reaction mechanisms of both PL and CL. In this case, a fluorogenic substrate A is converted
25 to a fluorescent product C by chemical reaction, and luminescence is generated by excitation of this product:



For analytical purposes, one of the reactants of the assay system that is capable of generating luminescence can
30 be attached to a molecule in order to "label" it specifically. The presence or absence of an observable label attached to one or more of the binding materials is then used as an indicator of the existence of an analyte of interest. A large body of experiments has been developed to
35 detect and quantitate trace amounts of pharmaceuticals, microorganisms, hormones, viruses, antibodies, nucleic acids

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and other proteins by such methods. In clinical diagnostics for instance, competitive and sandwich immunoassays using luminescence detection are now used on a routine basis ^{27, 28}.

In enzyme-mediated immunoassays, a molecule is labelled with an enzyme that catalyzes the luminescence reaction. Typical examples are the detection of immunoreagents labelled with Horse Radish Peroxidase (HRP) or Alkaline Phosphatase (ALP) which, in the presence of hydrogen peroxide and hydroxide ions, respectively facilitate the oxidation of luminol and dioxetanes and the hydrolysis of phosphate-containing reagents. Similarly, ALP has been used in CF assays to cleave a phosphate group from a fluorogenic substrate to yield a highly fluorescent product ²⁹.

Luminescence assay methods are widely used in the analysis of peptides, proteins, and nucleic acids. CL has been shown to be a highly sensitive detection method in both flow injection analysis (FIA) and high-performance liquid chromatography ³⁰⁻³², and it has also been employed in capillary electrophoresis (CE) ^{33, 34} for the detection of amino acids neurotransmitters ³⁵, rare-earth metal ions ³⁶ or labelled proteins ³⁷. However, it is in immunoassays that luminescence is the most commonly used detection method ^{27, 34, 38-43}.

Among prior art methods for the measurement of enzymatic reaction rate, US 4,621,059 discloses a method in which the light emitted by a luminescent substance flowing through a capillary column and reacting with an immobilised enzyme is collected through a plurality of optical fibers that are arranged along the longitudinal direction of the column in order to determine the enzyme activity or the quantity of analyte of interest from the distribution of the luminescence intensity.

US 5,624,850 describes a method for performing immunoassays in capillaries in which fluorescence is used to detect an analyte of interest in translucent capillaries

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having an inner diameter from $\sim 0.1 \mu\text{m}$ to 1.0mm . Similarly, homogeneous chemiluminescence immunoassays can be carried out, for example as described in US 5,017,473, in which a light absorbing material and a luminescent labelled tracer 5 are incubated with the analyte/anti-analyte complex, so that all the emitted light is absorbed by the light-absorbing material except that associated to the bound tracer.

A method is disclosed in US 5,585,069 in which two or more samples are processed in parallel in a system 10 comprising a plurality of wells that are connected by one or more channels to move a sample from one well to the other using mechanical or electrokinetic pumping. In this apparatus, the channels are simply used as connections between two wells, and are not used as reaction or detection 15 chambers.

One of the main difficulties associated with the use of μ -TASs is the proper mixing of reagents, due to the low Reynolds numbers of the flows. Another difficulty resides in the accurate timing of fluid entry, which is essential 20 for kinetics studies. In addressing these problems, the applicants have found that fluid entry into a reaction chamber (for example a microchannel) can be accurately be controlled by means of a hydrophobic gate.

The present application therefore provides, in one 25 aspect, apparatus comprising: at least one reaction chamber; at least one fluid inflow channel communicating with the or each reaction chamber; and gate means adapted to prevent passage of aqueous fluid through the fluid inflow channel(s) into the reaction chamber(s), until such fluid is acted upon 30 by a fluid entry force; wherein the gate means comprises at least a portion of the or each fluid inflow channel having a hydrophobic inner surface.

Preferably, the apparatus has a plurality of reaction chambers, which take the form of microchannels, each having 35 an associated fluid inflow channel. Alternatively, a

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plurality of microchannels may be served by a single inflow channel, feeding into a common conduit communicating with the microchannels. In some preferred embodiments, the fluid entry force is provided by aspiration means connected to a common conduit communicating with each microchannel at its end distal the inflow channel. In other embodiments, the apparatus comprises a rotatable support member and the fluid entry force is provided by centrifugal force upon rotation of the substrate. Conveniently, the support member may form the substrate of the microchannel apparatus, with the microchannels being arranged generally radially. Alternatively the rotatable support member may serve as a support for one or more devices having parallel microchannels.

15 The advantage of a common source of fluid entry force for all of the microchannels is that simultaneous filling may be ensured, the fluid samples being prevented from entering the microchannels by the hydrophobic gate means until the fluid entry force is applied. Furthermore, the degree of fluid entry force may also readily be controlled, to ensure rapid filling of the microchannels, and adequate mixing. The microchannels may also be emptied in an efficient and rapid manner, by application of an increased force to the fluid in the channels, for example by increasing the degree of aspiration, or by increasing the rate of rotation of the rotation support member. An exact end point of an assay may thereby be achieved. In many instances it is advantageous for the sample to be expelled before monitoring for bound target species.

30 If desired, a liquid reagent or a washing fluid may be supplied in a sealed cavity forming a reservoir, there preferably being one such reservoir per microchannel. The reservoirs may be arranged to communicate with their respective microchannels via normally closed valves, and may be caused to expel their contents through such valves when acted upon by respective pistons. Alternatively, there may

be a single reservoir, communicating via a normally closed valve with a common conduit feeding all microchannels.

Detection of target species with the microchannels may be achieved by conventional means. For example, to permit
5 electrochemical detection, preferred embodiments of apparatus are constructed so that at least a portion of the surface of the microchannel is formed of an electrically conductive material. This may for example be a conductive polymer material or an electrode. In some embodiments, at
10 least a portion of the microchannel walls may be formed of a semi-conductor material such as indium oxide. Preferably, the semi-conductor material is transparent. Alternatively, detection may be achieved by luminescence or fluorescence means, in which case an electromagnetic radiation detector,
15 such as a photodiode or a photomultiplier, is provided.

One particular advantage of the invention is that chemical reagents may be immobilised on the inner surfaces of the microchannels, thus providing the possibility of ELISA - type assays in a μ -TAS-type system. A number of
20 different types of reagent may be attached to the microchannel walls, for example oligonucleotides, polypeptides, proteins (such as enzymes), or other natural or synthetic molecules. Conveniently, these may be adsorbed onto the surface of the microchannel walls, or covalently
25 linked thereto, (for example by means of amide bond formation with succinimide), or electrostatically linked thereto (for example by means of a crosslinker such as polylysine). The inner surface of the microchannel and/or of the fluid inflow channel may also be provided with
30 chemically functional groups formed by chemical or physical treatment.

The invention also extends to a method of manufacturing an apparatus as defined above, comprising the following steps which may be performed in either order or
35 simultaneously: forming at least one reaction chamber; and forming at least one fluid inflow channel communicating with

the reaction chamber(s), at least a portion of the or each fluid inflow channel having a hydrophobic inner surface adapted to act as gate means to prevent passage of fluid through the fluid inflow channel into the reaction chamber(s) until such fluid is acted upon by a fluid entry force.

For ease of fabrication, the apparatus is preferably formed in two main parts: a substrate in which the microchannels (and possibly also the inflow channels) are formed as depressions (for example by injection moulding, hot embossing, photoablation, casting or polymerisation on a mould); and an overlying layer applied over the substrate and over the depressions, to form the microchannels (and optionally also the inflow channels). In embodiments in which the inflow channels are not produced in the substrate they may, for example, be produced by drilling through a laminated overlying layer using a laser, or by depositing above the inlet of the reaction chamber a joint made of a hydrophobic material such as polydimethylsiloxane (PDMS).

The apparatus may be formed from any suitable material, for example, ceramics, glass, semiconductors, polymers, or combinations thereof. In a particularly preferred embodiment, both the substrate and lamination layer are formed of polymer material, which not only permits ready formation of the microchannels (for example by photoablation), but also allows the two components to be fused together by a thermal lamination technique. For this purpose, it is preferred that at least one of the polymers is of a material which has a relatively low melting point, for example polyethylene with a melting point of under 200°C. The lamination layer may with advantage be of an elastomeric material, such as polydimethyl siloxane (PDMS). In apparatus for use in conjunction with optical detection means, it is preferred that the lamination layer be formed of a substantially transparent material, and the substrate of a substantially opaque material (such as a ceramics material or a carbon-filled polymer).

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In another aspect, the invention extends to a method of operating an apparatus as defined, comprising the steps of: placing at least one sample of an aqueous solution under test at the end of at least one fluid inflow channel distal
5 at least one reaction chamber; causing the sample to enter the reaction chamber(s) via the fluid inflow channel(s) by applying a fluid entry force; and monitoring the sample in the reaction chamber(s) for the presence or concentration of a target substance.

10 In embodiments of apparatus having aspiration means, the fluid entry force is preferably applied by activating the aspiration means to apply reduced pressure to the microchannels for a period of time in the range 0.1 to 100s. In order to evacuate the microchannels, the aspiration means
15 may then be activated to provide an even lower pressure to the microchannels, optionally in conjunction with the supply of washing fluid from a reservoir.

In embodiments of apparatus constructed using rotatable substrates or supports, the fluid entry force is preferably
20 applied by rotating the substrate or support at an angular velocity in the range 1 to 1,000 revolutions per minute for a period of time in the range 1 to 100s. The microchannels may then be evacuated by rotating the substrate or support at an increased angular velocity, in the range 10 to 100,000
25 revolutions per minute, for a period of time in the range 1 to 100s.

The invention is hereinafter described in more detail by way of example only, with reference to the accompanying drawings, in which:

30 Fig 1 is a schematic cross section of an embodiment of apparatus according to the invention illustrated a) after deposition of an aqueous sample drop on the hydrophobic gate, and b) after sample loading;

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Fig 2 is a schematic plan view of an embodiment of apparatus according to the invention, illustrating the steps of parallel sampling, loading and washing, achieved by aspiration;

5 Fig 3 is a schematic plan view of an alternative embodiment of apparatus according to the invention, in which parallel filling and washing step are achieved by centrifugal force;

Fig 4 is a schematic plan view of a further embodiment
10 of apparatus according to the invention, in which the solution is loaded by slight aspiration and washed by strong aspiration;

Fig 5a is a partial plan view of an embodiment of apparatus according to the invention, incorporating a fluid
15 reservoir adjacent the fluid inflow channel;

Fig 5b consists of two vertical cross sections of the Fig. 5a apparatus together with an associated piston, illustrating the action of the piston in penetrating the sealed reservoir and expelling its contents through a valve
20 into the reaction chamber;

Fig 6 is a top plan representation of an embodiment of apparatus according to the invention manufactured by UV-Laser photoablation of a polycarbonate compact disk, the embodiment being constructed substantially as the apparatus
25 of Fig 3;

Fig 7 is a calibration curve obtained from a fluorescence imager using ALP-DDi solution in the microchannel of an embodiment of apparatus according to the invention;

30 Fig 8 is a graph illustrating fluorescence results indicating the level of binding between DDi-ALP in a test involving use of two microchannels of apparatus according to

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the invention, one incubated solely with BSA, and the other with BSA and anti-DDi (noted as BSA + Ab in the figure);

Fig 9 is a fluorescence image obtained using apparatus of the type illustrated in Fig 6, in which different 5 microchannels were incubated with different concentrations of DDi-ALP;

Fig 10 is a graph illustrating the intensity of fluorescence obtained from different concentrations of ALP-DDi in an apparatus of the type illustrated in Fig 6, having 10 antibody-coated microchannels;

Fig 11 is a graph illustrating variation of fluorescence intensity with time from an incubation of ALP-DDi in an embodiment of apparatus according to the invention, having at least one antibody coated microchannel; 15 and

Fig 12 is a graph illustrating fluorescence intensity (indicating bound ALP-DDi) in a competitive immunoassay with D-Dimer utilising an embodiment of apparatus according to the invention, having at least one antibody-coated 20 microchannel.

The microchannel devices of Figs 1 to 6 are produced by UV-Laser photoablation of commercially available polymers such as PET or polycarbonate. The photoablation procedure is performed in known fashion, for example as described 25 previously by the present applicants⁴⁴. Briefly, a polymer sheet is rinsed with distilled water and ethanol and then mounted on an X,Y machining stage (Microcontrol, France). UV-Laser pulses (193 nm) (Lambda Physik LPX 205 i, Germany) are then fired at the polymer substrate target through a 30 photomask and a 10:1 lense with a frequency of 50 Hz at 200 mJ/pulse, corresponding to a fluence per pulse of 1 J/cm² on the surface. During the photoablation process, the polymer substrate is moved horizontally with a X,Y stepping motor (Microcontrol, France) at a speed of 0.2 mm/s resulting in

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linear channels 22 mm long. The microchannels are typically between 1 and 1,000 μ m in width, and in this example are approximately 100 μ m wide. The depth of the channels was fixed at 40 μ m, by controlling the number of laser pulses 5 used (each pulse photoablates approximately 150 nm). The channels are then sealed by thermal lamination of a layer of polyethylene over the base polymer sheet, the channels then exhibiting a trapezoidal shape in which three walls are composed of the substrate polymer (PET or Polycarbonate) and 10 the top is composed of the lamination (Polyethylene). Fluid inflow channels (or "gates") (1) are opened either by firing enough laser pulses or are mechanically drilled through the hydrophobic lamination layer. The gates, which may have a diameter between 10 μ m and 10mm, have hydrophobic inner 15 surfaces due to the nature of the polymer, and therefore inhibit passage of aqueous fluids.

The precise arrangement of microchannels is not crucial to the operation of the invention, though two general geometries have been developed and tested by the applicants 20 and proved to be of benefit. In the first of these, a plurality of microchannels are arranged parallel to each other, conveniently on a generally rectangular substrate. The inflow channel "gates" of the various microchannels are aligned with each other, to permit rapid and efficient 25 loading with test solutions from a linear multiple pipette device (see Fig 2). In the second configuration, the microchannels are arranged radially on a generally circular substrate, either with the inflow channel gates towards the centre of the circle and the opposite (outflow) ends of the 30 microchannels towards the circumference (Figs 3 and 6), or vice versa (Fig 4).

A number of different means may be employed to provide the fluid entry force, of which the preferred means are aspiration and centrifugal force. In the apparatus of Fig 35 2, a common conduit (3) is supplied at the outflow ends of the microchannels (2), to which a reduced pressure is applied during operation of the device, to draw fluid into

the microchannels through the fluid inflow gates. As illustrated in the last representation in Fig 2, the aspiration means may also be utilised to supply a stronger aspirating force in order to expel the microchannel contents to a drain, optionally in conjunction with the supply of a washing fluid. The apparatus illustrated in Fig 4 operates in a similar fashion, with aspiration being applied to the common outflow drain (6). In the apparatus illustrated in Figs 3 and 6, fluid is compelled to pass through the fluid inflow gates and into the microchannels by spinning the substrate to produce centrifugal force. In the illustrated arrangement, each microchannel has its own drain (7).

Typically, in an aspiration driven device (as in Figs 2 and 4) a 2 μ l sample is placed with a pipette on each gate (1). The solution is then loaded into the microchannel by a brief aspiration from the common conduit (3;6). This technique ensures homogeneity of the solution over the whole microchannel. After incubation, the microchannel is aspirated and rinsed three times with 2 μ l. It is worth noting that the washing solution volume is much larger than that of the microchannel (about 100 nl) thus ensuring efficient washing. Using devices driven by centrifugal pressure, the filling and washing procedures may be achieved by placing 2 μ l of solution over each gate (1). Slow rotation results in loading of the sample into the microchannel(s), and faster rotation is subsequently used to expel the sample from the microchannel(s).

Fig 5 illustrates an optional modification of apparatus according to the invention, in which each microchannel has an associated fluid reservoir (10) formed by a sealed cavity situated adjacent the fluid inflow gate (1). The reservoir communicates with microchannels (2) by means of a normally closed valve (12), which comprises valve member (13) which may be deformed under pressure into depression (14). Reservoir (10) is capped by seal (15), which may be broken by downward pressure applied by piston (11), which is profiled to be a close fit within reservoir (10). Downward

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movement of piston (11) within reservoir (10) increases the fluid pressure within the reservoir, thus opening valve (12) and allowing fluid from the reservoir to enter the microchannel. Depending upon the requirements of any particular assay, the reservoir may either be filled with a reagent or with wash fluid.

By way of example, various tests were carried out to establish the utility of apparatus according to the invention in performing an immunoassay for D-Dimer. D-Dimer is used as a diagnostic indicator in thromboembolic events: deep vein thrombosis and pulmonary embolism can be diagnosed by monitoring D-Dimer concentration in blood. In the past, the most reliable assay of D-Dimer have been performed by ELISA techniques, for example the "Asserachrom D-Di" of Diagnostica Stago. However, standard ELISA techniques are not suited for emergency situations, and alternative membrane-based techniques have been developed which use colour based detection systems ⁴⁸. However, these suffer from the disadvantage that the detection mechanism is too subjective.

In the present tests, the detection of the enzyme was effected by a chemifluorescent substrate solution (VCR, Amersham). This system is based on the fluorescent detection of the AttoPhos substrate hydrolysed by ALP. The microchannels were then exposed to a Fluorescence Imager screen (MP840, Molecular Dynamics) and every channel was read for 1 minute. The image was then quantified using Image Quant software (Molecular Dynamics). The calibration of the enzyme in the microchannel was achieved by mixing the substrate solution with different concentrations of enzyme and incubating for 5 minutes. The microchannels were then filled with the mixtures and analysed with the fluorescence imager. In the actual tests, the enzyme was immobilised on the surface of the microchannels, and the VCR solution was added to the channels with fluorescence being measured 5 minutes later.

Immobilisation of the proteins was achieved by physisorption for 1 hour at room temperature. The mouse IgG antibody (Serbio, France) was immobilised by placing either 10 or 100 $\mu\text{g/ml}$ in the microchannel, followed by incubation 5 for 1 hour in a wet chamber. The surface was then washed with PBS and 20 % Tween (Tween/Water :0.2 ml/L, Fischer Germany), and blocked for 1 hour with a solution of 50 $\mu\text{g/ml}$ of heat shocked BSA (Sigma, USA) in the washing buffer solution. After another washing step, the channels were 10 individually filled with the antigen solution. After five minutes (except for the kinetic experiment, where other periods are specified below), the microchannels were rinsed and a solution of 10 $\mu\text{g/ml}$ of alkaline phosphatase labelled antigen (ALP-DDi) was introduced and rinsed again after five 15 minutes.

Fluorescence dependence on enzyme concentration after 5 minutes of incubation is presented graphically in Figure 7. The detection limit is reached in the range of 1 ng.ml^{-1} . The non-linear detection range is due to the fact that the 20 product of the hydrolysis is not highly soluble and may precipitate on the surface at higher concentrations. Nevertheless, this system can be used for quantifying the enzyme concentration in the microchannel.

In order to study the activity of the adsorbed 25 antibodies in the microchannel, two different incubation procedures were undertaken. Firstly, a few channels were incubated only with BSA. Secondly, some further channels were incubated with Ab and then with BSA. Every channel was then filled with the DDi-ALP, incubated for 1 hour and 30 washed by aspiration following the procedure described above. The fluorescence intensity of each channel was then measured and the results are presented in Figure 8. The channels incubated only with BSA exhibited a low fluorescence which is not significantly different from that 35 of the polymer substrate itself. In contrast the channels incubated with the antibodies were much more fluorescent, thereby demonstrating that DDi-ALP was adsorbed on the

antibodies. This experiment shows that some of the adsorbed antibodies are still active on the surface and that BSA is an effective blocker against the non-specific adsorption of the DDi-ALP complex.

5 Figure 9 shows the fluorescence of the substrate in the channels after adsorption of different concentrations of ALP-DDi on the $10 \mu\text{g}.\text{ml}^{-1}$ adsorbed antibodies. The fluorescence intensity of the microchannel lines clearly shows the gradient of concentration in the different
10 microchannels. The relative intensity of every microchannel is shown graphically in Figure 10. Saturation of channels is reached at about $30 \mu\text{g}/\text{ml}$.

Figure 11 shows the fluorescence intensity of microchannels that have been incubated for different periods
15 of time. For short incubation times ($<5 \text{ min}$), the intensity grows linearly, showing that the antigens are very quickly captured by the antibodies. It is thought that all the antigens have still not reached the surface by diffusion. This first slope approximately follows the diffusion of the
20 molecules to the walls. The molecules then react rapidly and the reaction becomes quasi diffusion-controlled. After 5 minutes of incubation, the reaction is controlled by slower kinetics driven by two different phenomena. Firstly, large molecules diffuse much more slowly and therefore reach the
25 surface after a long time. In this case, the molecules can be partially degraded fibrin products, of which the molecular weight can be larger than 1000 kD . Secondly, there is a tendency for non-specific adsorption; such reactions are much slower than immunological recognition and are
30 driven by electrostatic or hydrophobic interactions that require reorganisation at the molecular level. This type of non specific adsorption may therefore be excluded by short incubation times.

Figure 12 shows the fluorescence dependence of the D-
35 Dimer concentration after a competitive immunoassay. In the low concentration range, most of the immobilised antibody

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sites are not occupied by the DDi, allowing the DDi-ALP to be present in a large amount and therefore to hydrolyse more fluorescent substrates. At concentrations higher than 1000 ng.ml⁻¹, D-Dimer molecules are present on most of the 5 antibody sites and therefore only a few sites are available for DDi-ALP. The central part of the concentration range (100-1000 ng.ml⁻¹) shows the strong concentration dependence of the system, the two orders of magnitude detection range being in the range of interest for diagnostics applications 10 ⁴⁹.

These experiments demonstrate the feasibility of ELISA techniques in microchannels. Benefitting from fast equilibration times and rapid filling and rinsing procedures, the time taken to complete an assay (including 15 calibration) may be reduced to less than 10 minutes, compared to a typical time of 3 hours for an ELISA in a microtiter plate. Hundreds of microchannels may be provided on a substrate, if desired, and the ability to ensure simultaneous filling provides the possibility of highly 20 efficient parallel assays.

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CLAIMS

1. Apparatus for performing chemical assays involving aqueous fluids, the apparatus comprising: at least one reaction chamber; at least one fluid inflow channel
5 communicating with the or each reaction chamber; and gate means adapted to prevent passage of aqueous fluid through the fluid inflow channel(s) into the reaction chamber(s), until such fluid is acted upon by a fluid entry force; wherein the gate means comprises at least a portion of the
10 or each fluid inflow channel having a hydrophobic inner surface.

2. Apparatus according to claim 1, wherein the reaction chamber comprises a microchannel having at least one dimension in the range 1 to 1,000 μm .

15 3. Apparatus according to claim 1 or claim 2, wherein the fluid inflow channel is formed in a substrate at least a portion of which is composed of a hydrophobic material.

4. Apparatus according to any of claim 1 or claim 2, wherein the fluid inflow channel is formed in a substrate at
20 least a portion of which is physically or chemically treated to render it hydrophobic.

5. Apparatus according to any preceding claim, wherein the fluid inflow channel has a cross-sectional area in the range 10 μm^2 to 1,000 mm^2 .

25 6. Apparatus according to any preceding claim, wherein the fluid inflow channel is moulded to be complementary in shape to a standard pipette (for example of the Eppendorf^(RTM) type).

7. Apparatus according to any preceding claim,
30 wherein the fluid entry force is provided by piston pressure.

8. Apparatus according to any preceding claim, comprising a plurality of separate reaction chambers, each communicating with an inflow channel and associated gate means.
- 5 9. Apparatus according to claim 8, wherein each reaction chamber is provided with a separate inflow channel.
- 10 10. Apparatus according to claim 8, wherein there is one inflow channel forming a common conduit to all reaction chambers.
- 10 11. Apparatus according to claims 8, 9 or 10, as
pendant to claim 2, wherein each microchannel communicates, at its end distal the inflow channel, with a common conduit, the common conduit being connected to aspiration means adapted selectively to apply reduced
15 pressure to the conduit and thus to draw fluid through the microchannel in operation.
12. Apparatus according to claim 10 or claim 11, wherein the common conduit has a cross-sectional area in the range 0.01mm^2 to 25cm^2 .
- 20 13. Apparatus according to any preceding claim, wherein the microchannels are arranged generally parallel to each other.
14. Apparatus according to claim 13, as pendant to any of claims 10 to 12, wherein the microchannels are
25 arranged generally perpendicularly to the common conduit.
15. Apparatus comprising a plurality of apparatuses according to claim 14 mounted together on a tape.
16. Apparatus according to claim 11, comprising a substantially circular substrate the microchannels being
30 arranged substantially radially, each having its inflow channel towards the circumference of the circle and its

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opposite end communicating with a central chamber connected to said aspiration means.

17. Apparatus according to claim 8 or claim 9, comprising a substantially circular substrate the 5 microchannels being arranged substantially radially, the inflow channel(s) being disposed towards the centre of the circle, and each microchannel having a waste chamber at the opposite end towards the circumference of the circle.

18. Apparatus according to claim 16 or claim 17, 10 wherein the thickness of the substantially circular substrate is in the range 50 to 5,000 μ m.

19. Apparatus according to claim 16, wherein the circular substrate is rotatable, and the fluid entry force is provided by centrifugal pressure as the substrate is 15 caused to rotate.

20. Apparatus comprising a plurality of apparatuses according to claim 13 or claim 14 disposed on a rotatable support member, and wherein the fluid entry force is provided by centrifugal pressure as the support member is 20 caused to rotate.

21. Apparatus according to any preceding claim, wherein the or each reaction chamber is provided, proximate thereto, with a sealed cavity forming a reservoir filled with an aqueous fluid.

25 22. Apparatus according to claim 21, wherein the reservoir communicates with the reaction chamber via a normally closed valve, which may be caused to open by the application of increased pressure to the aqueous fluid within the cavity.

30 23. Apparatus according to claim 22, further comprising a piston member having an outer profile shaped to fit within the cavity, the cavity being capped by a

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breakable seal, which, in operation, is broken by the piston, wherein movement of the piston into the cavity provides the increased pressure necessary to urge the aqueous fluid from the cavity via the valve into the
5 reaction chamber.

24. Apparatus according to any preceding claim, wherein at least a portion of the surface of the reaction chamber is formed of an electrically conductive material, and the apparatus further comprises electrical detection
10 circuitry connected to said conductive portion, to enable detection of a target species within the reaction chamber by electrochemical means.

25. Apparatus according to claim 24, wherein said conductive portion is formed of a conductive polymer
15 material.

26. Apparatus according to claim 24, wherein said conductive portion is formed by an electrode.

27. Apparatus according to claim 26, wherein said electrode is of a semiconductor material.

20 28. Apparatus according to claim 27, wherein said semiconductor material is substantially transparent.

29. Apparatus according to claim 28, wherein the semiconductor material is indium oxide.

30. Apparatus according to any of claims 1 to 23,
25 further comprising electromagnetic radiation detection means adapted to detect radiation emitted by a target species in the reaction chamber.

31. Apparatus according to claim 30, wherein the detection means comprises at least one photodiode or at
30 least one photomultiplier array arranged along at least a portion of the reaction chamber.

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32. Apparatus according to any preceding claim, wherein a chemical reagent is immobilised on at least a portion of the inner surface of the reaction chamber, the reagent being adapted to interact with a target species
5 whose presence or concentration is to be determined.

33. Apparatus according to claim 32, wherein the reagent comprises an oligonucleotide, a polypeptide, a protein or another natural or synthetic molecule.

34. Apparatus according to claim 32 or claim 33,
10 wherein the reagent is adsorbed onto said inner surface of the reaction chamber.

35 Apparatus according to claim 32 or claim 33, wherein the reagent is covalently attached to said inner surface of the reaction chamber.

15 36. Apparatus according to claim 35, wherein the covalent bonding is achieved via a succinimide bonding agent.

37. Apparatus according to claim 32 or claim 33, wherein the reagent is electrostatically attached to said
20 inner surface of the reaction chamber via a crosslinker.

38. Apparatus according to claim 37, wherein the crosslinker is polylysine.

39. Apparatus according to any preceding claim, wherein at least a portion of the inner surface of the
25 reaction chamber and/or of the fluid inflow channel is provided with chemically function groups formed by chemical or physical treatment of the surface.

40. Apparatus according to any preceding claim, comprising a substrate, in which the reaction chamber and/or
30 the fluid inflow channel are formed as a depression(s), the

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reaction chamber and/or the fluid inflow channel being sealed by an overlying layer applied over the substrate.

41. Apparatus according to claim 40, wherein the substrate and overlying layer are formed of polymer materials, the melting point of at least one of the materials being sufficiently low to permit the substrate and overlying layer to be sealed together by thermal lamination.

42. Apparatus according to claim 41, wherein said at least one material is polyethylene.

10 43. Apparatus according to claim 40 or claim 41, wherein the overlying layer is formed of an elastomeric material.

44. Apparatus according to claim 43, wherein the elastomeric material is polydimethylsiloxane (PDMS).

15 45. Apparatus according to claim 40, as appendant to claim 30 or 31, wherein at least a portion of the substrate is formed of a substantially opaque material and the overlying layer is formed of a substantially transparent material.

20 46. Apparatus according to claim 45, wherein the substantially opaque material comprises a carbon-filled polymer or a ceramics material.

47. A method of manufacturing an apparatus according to any of claims 1 to 46, comprising the following steps which may be performed in either order or simultaneously:
forming at least one reaction chamber; and forming at least one fluid inflow channel communicating with the reaction chamber(s), at least a portion of the or each fluid inflow channel having a hydrophobic inner surface adapted to act as
30 gate means to prevent passage of fluid through the fluid inflow channel into the reaction chamber until such fluid is acted upon by a fluid entry force.

- 30 -

48. A method according to claim 47, wherein the apparatus is formed from polymeric material.

49. A method according to claim 48, wherein the apparatus is formed by injection moulding, hot embossing,
5 photoablation, casting, or polymerisation on a mould.

50. A method according to claim 48 or claim 49, comprising the steps of forming a substrate having at least one depression therein, and applying an overlying layer over the substrate to seal the or each depression so as to form
10 at least one fluid inflow channel and/or at least one reaction chamber.

51. A method according to claim 50, wherein the overlying layer is sealed with the substrate by thermal lamination.

15 52. A method according to claim 47, wherein at least a part of the apparatus is formed of a ceramics material, glass, a conductor or a semi-conductor material.

53. A method of operating an apparatus according to any of claims 1 to 46, comprising the steps of: placing at
20 least one sample of an aqueous solution under test at the end of at least one fluid inflow channel distal at least one reaction chamber; causing the sample to enter the reaction chamber(s) via the fluid inflow channel(s) by applying a fluid entry force; and monitoring the sample in the reaction
25 chamber(s) for the presence or concentration of a target substance.

54. A method according to claim 53, wherein the sample(s) is caused to exit the reaction chamber(s) before the reaction chamber(s) or the expelled sample is monitored
30 for the presence or concentration of a target substance.

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55. A method according to claims 53 or 54, wherein the or each sample is applied by means of a pipette, a syringe, or an electrically operated injector.

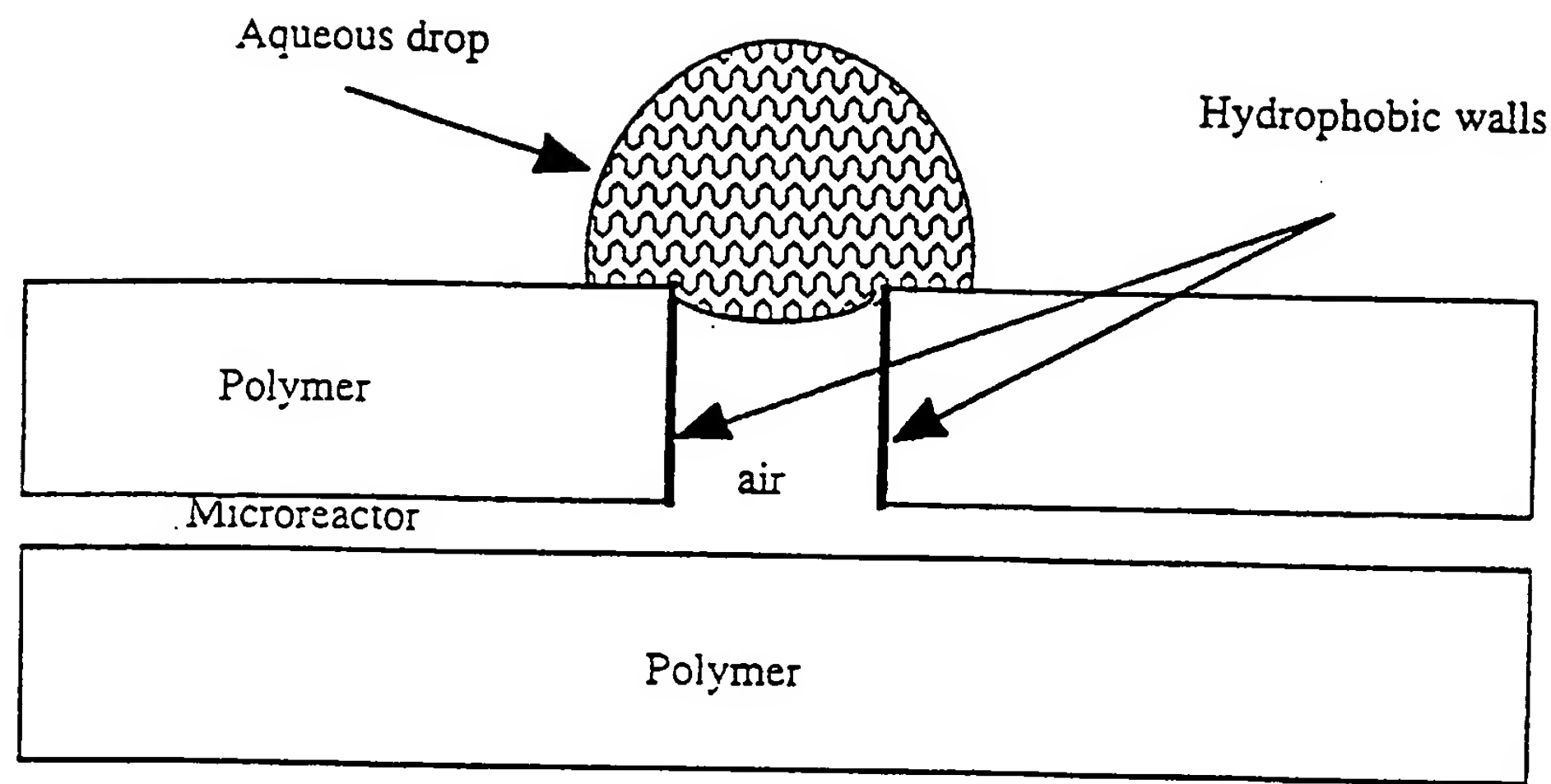
56. A method according to any of claims 53 to 55, for
5 operating an apparatus according to claim 11 or any claim
pendant thereto, wherein the fluid entry force is provided
by aspiration means, the aspiration means being activated to
apply reduced pressure to the or each reaction chamber for
a period of time in the range 0.1 to 100s.

10 57. A method according to any of claims 53 to 55, for
operating an apparatus according to either claim 19 or claim
20, or any claim pendant thereto, wherein the fluid entry
force is provided by spinning the substrate or the support
member at an angular velocity in the range 1 to 1,000
15 revolutions per minute for a period of time in the range 1
to 100s.

58. A method according to claim 57, wherein the sample
is expelled from the reaction chamber by spinning the
substrate at a greater angular velocity, in the range 10 to
20 100,000 revolutions per minute, for a period of time in the
range 1 to 100s.

59. A method according to any of claims 53 to 55, for
operating an apparatus according to claim 7 or any claim
pendant thereto, wherein the fluid entry force is provided
25 by piston pressure.

a)



b)

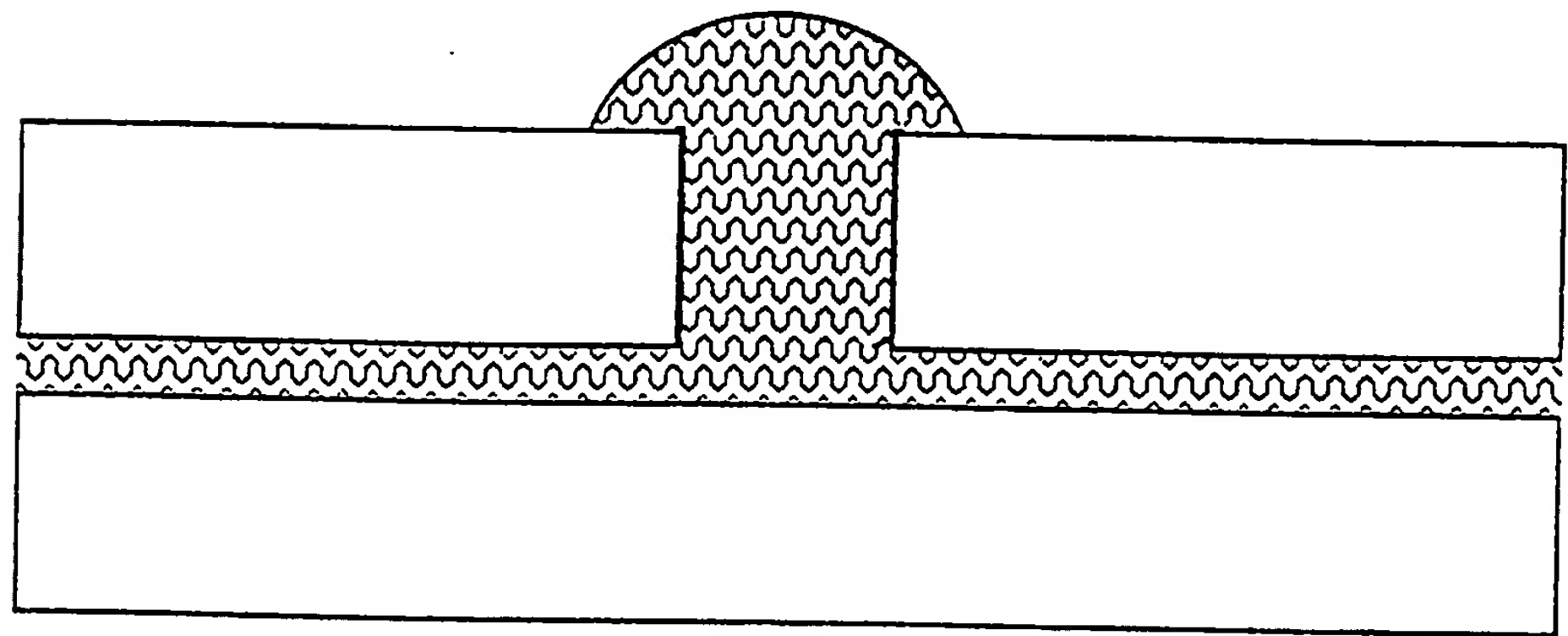


FIG 1

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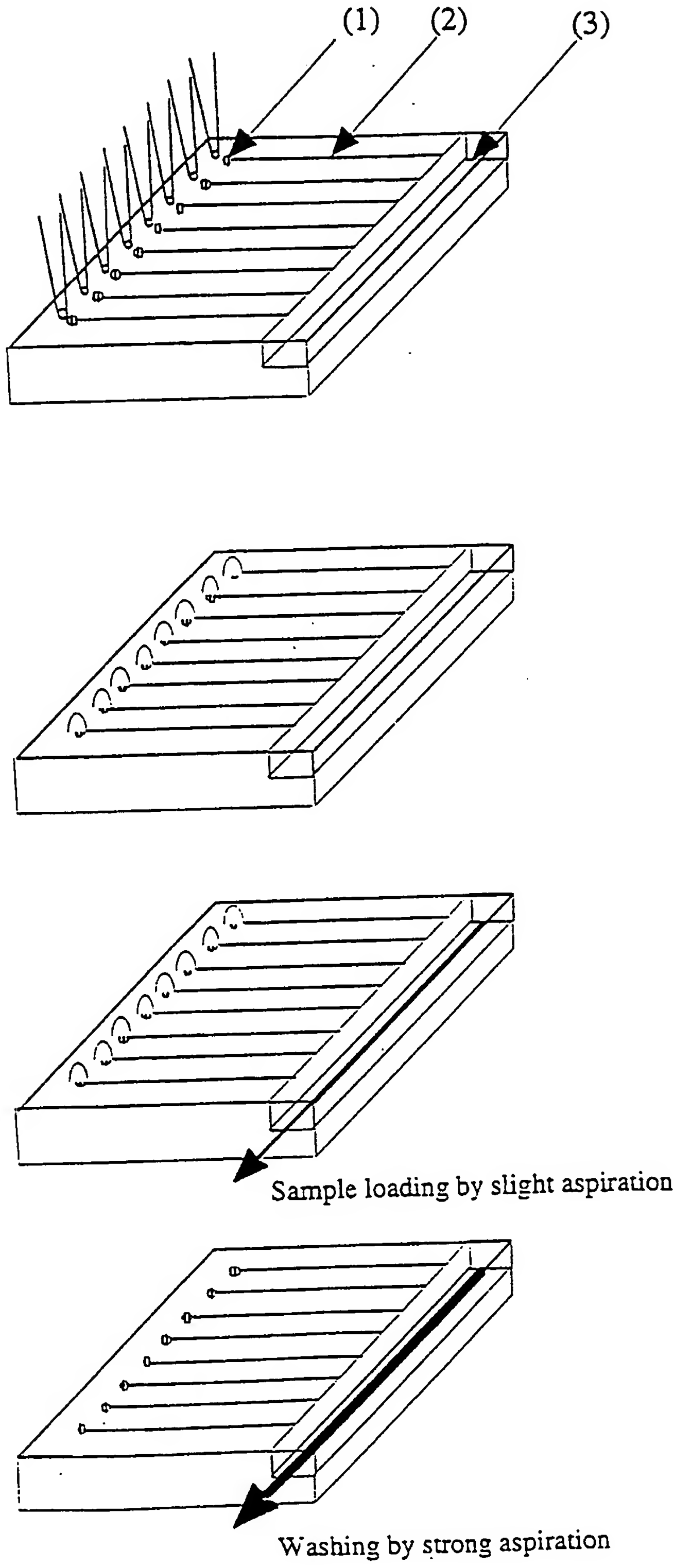


FIG 2

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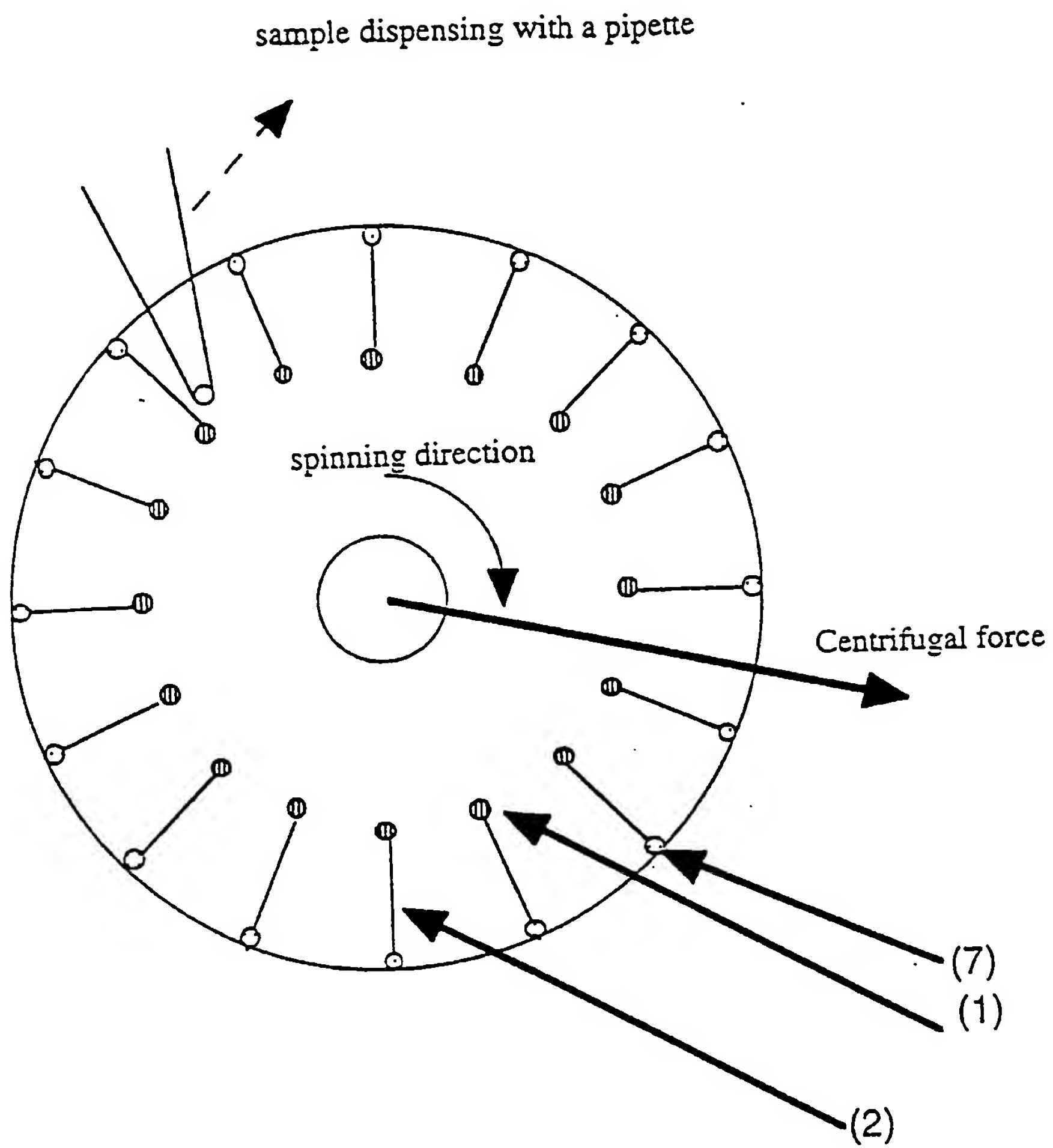


FIG 3

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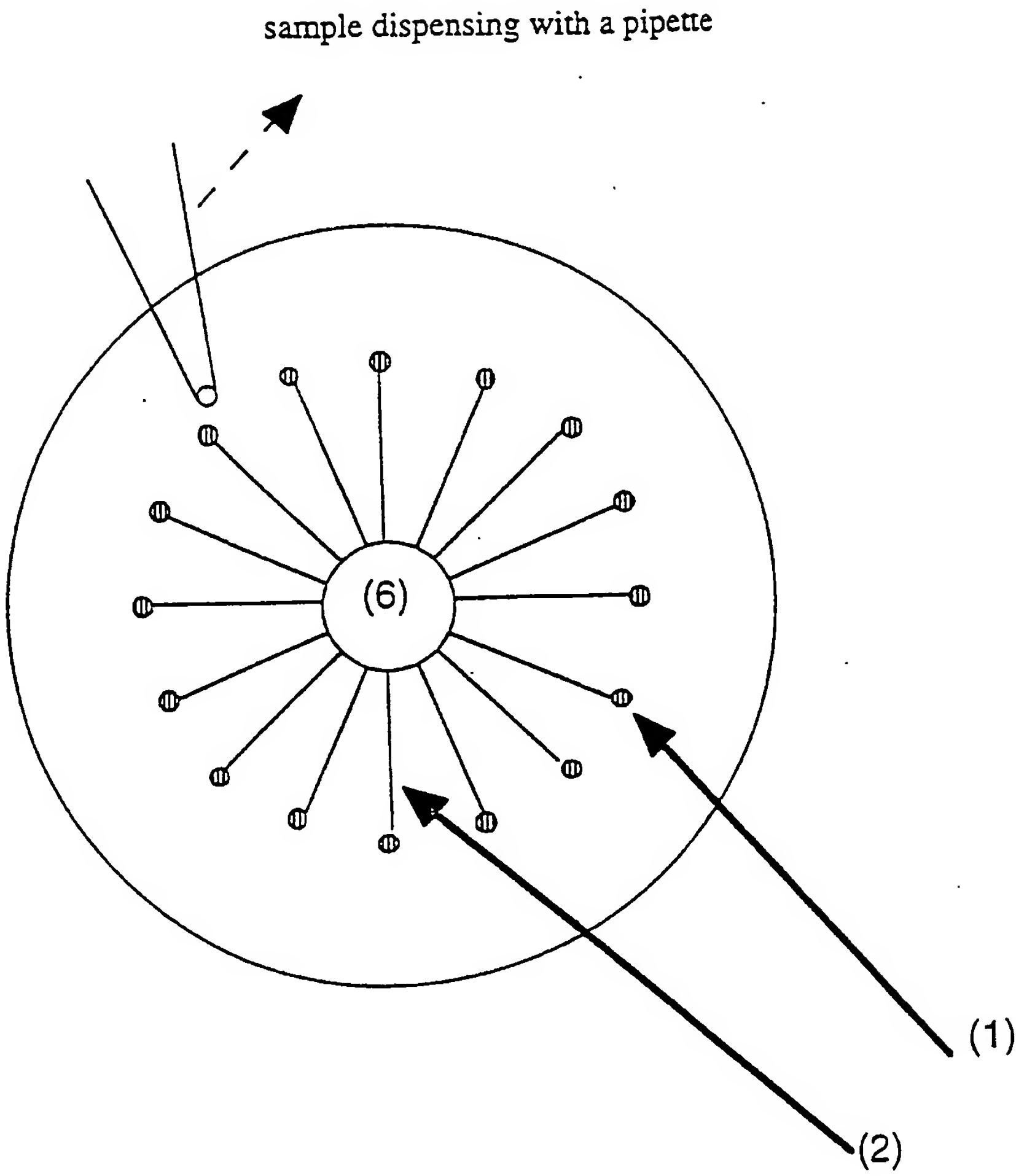


FIG 4

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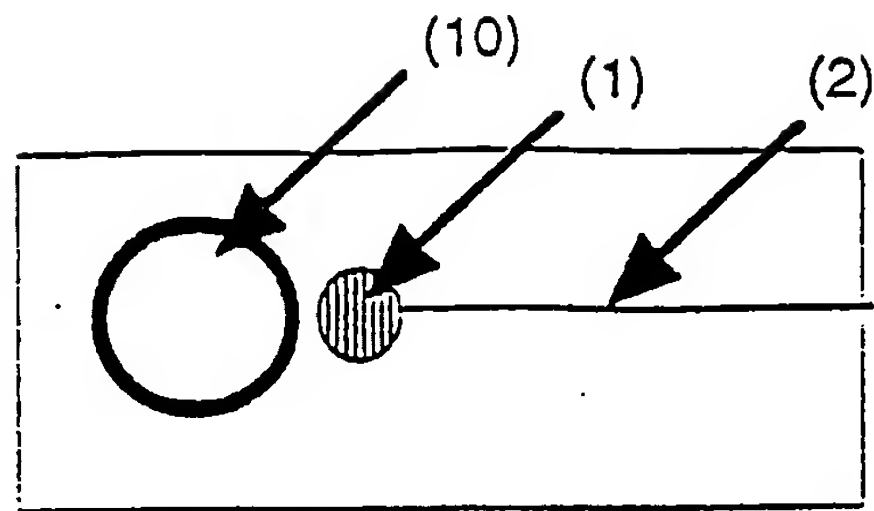


FIG 5a

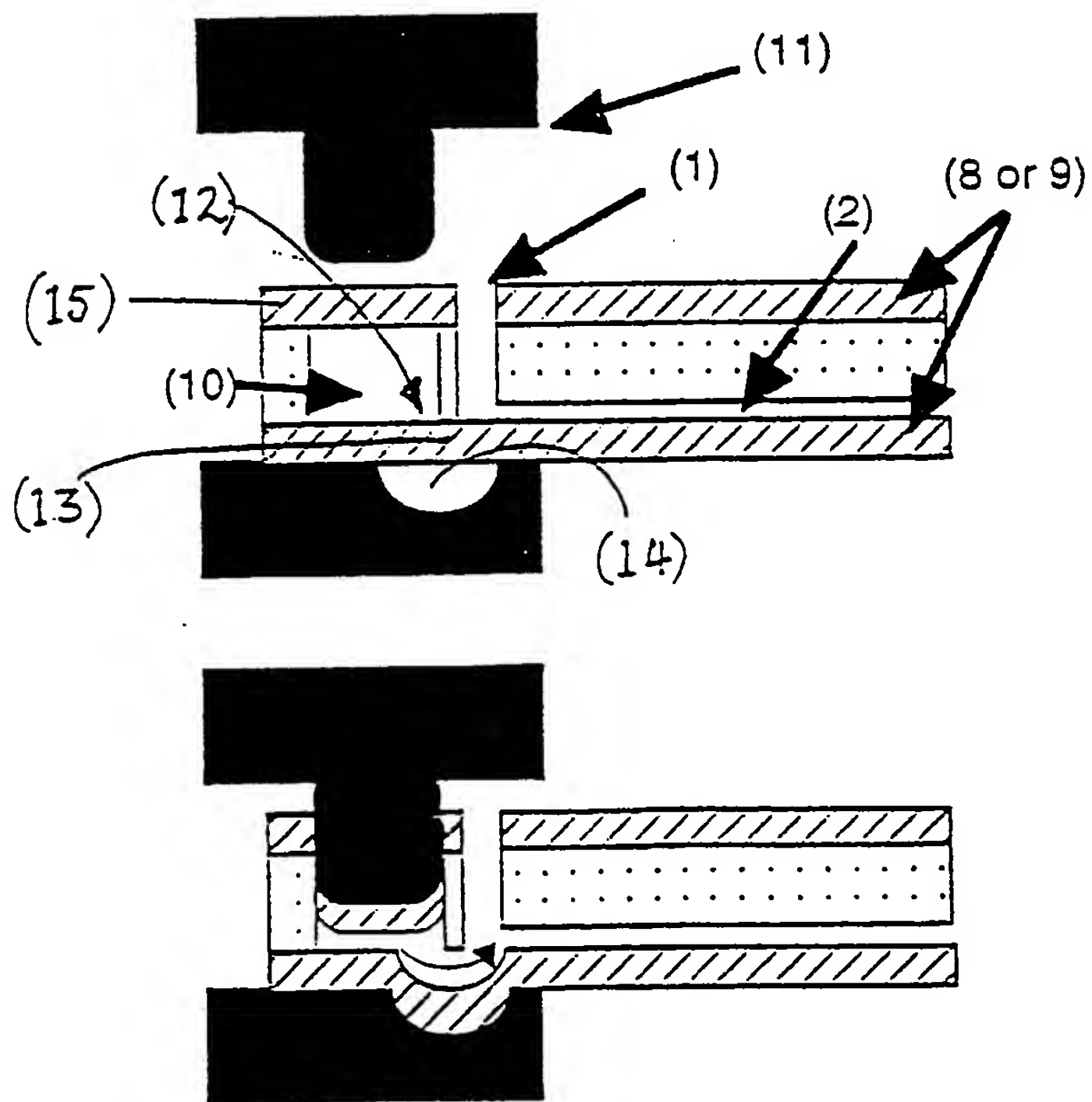
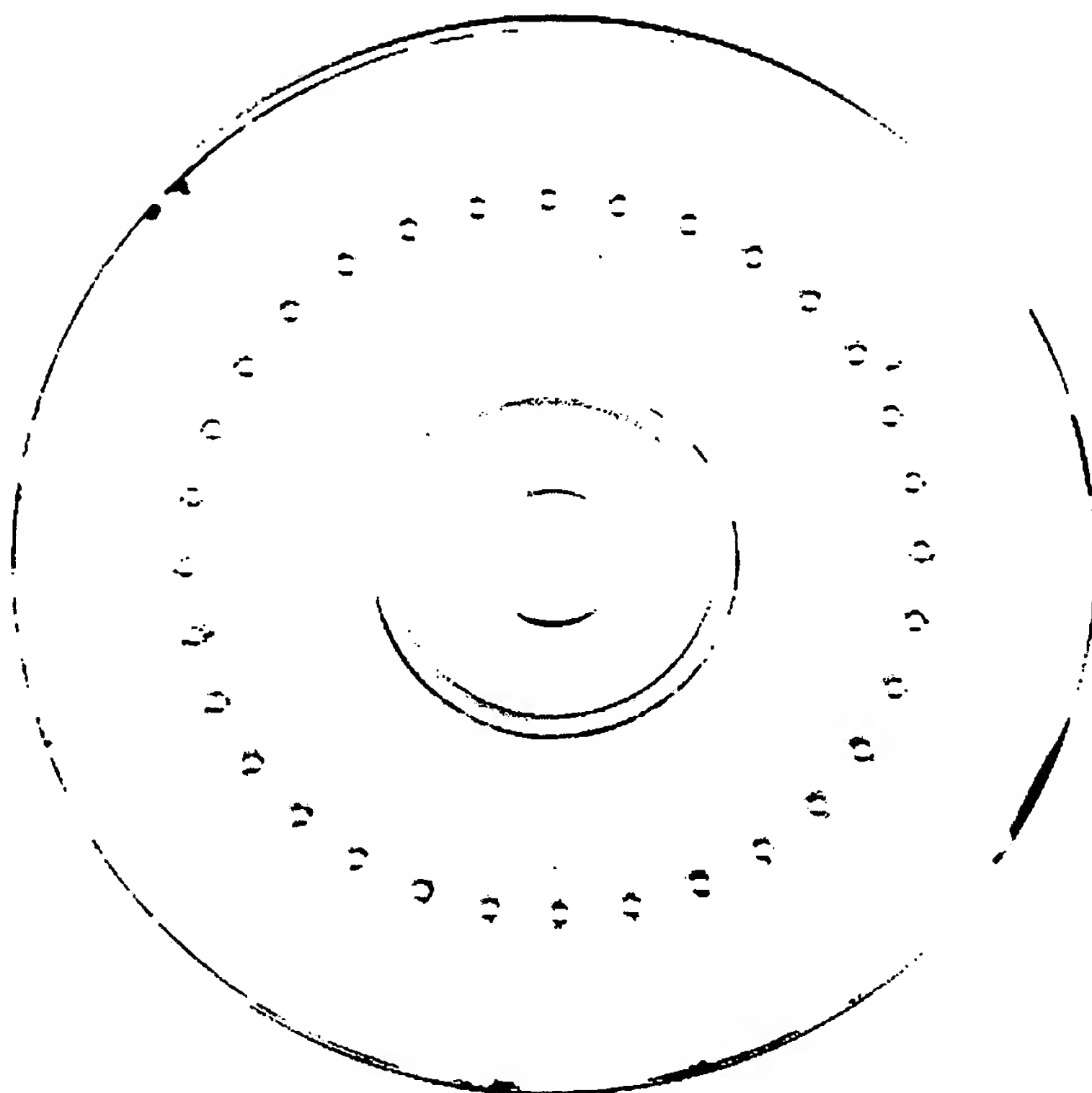


FIG 5b

Figure 6:



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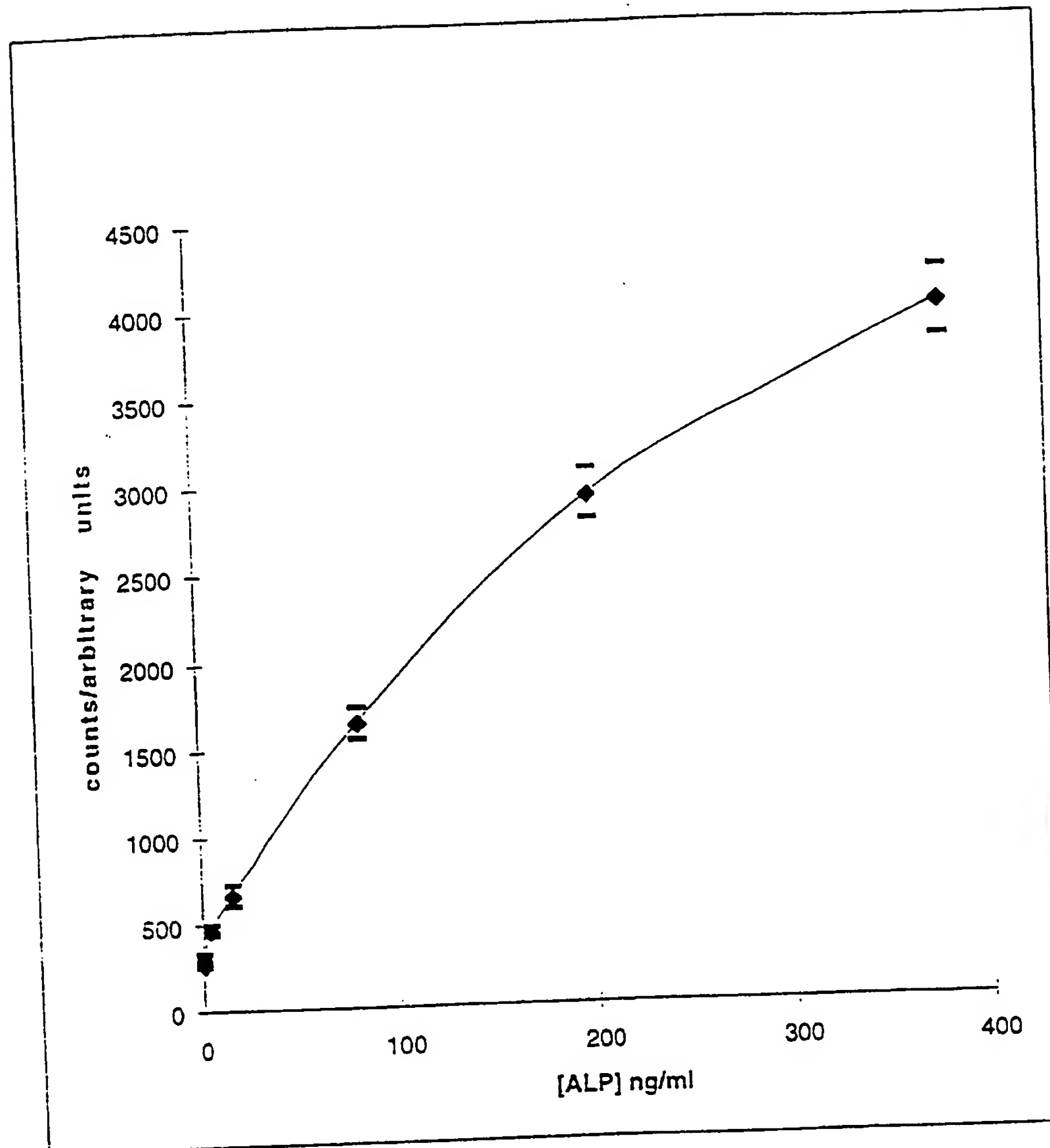


FIG 7

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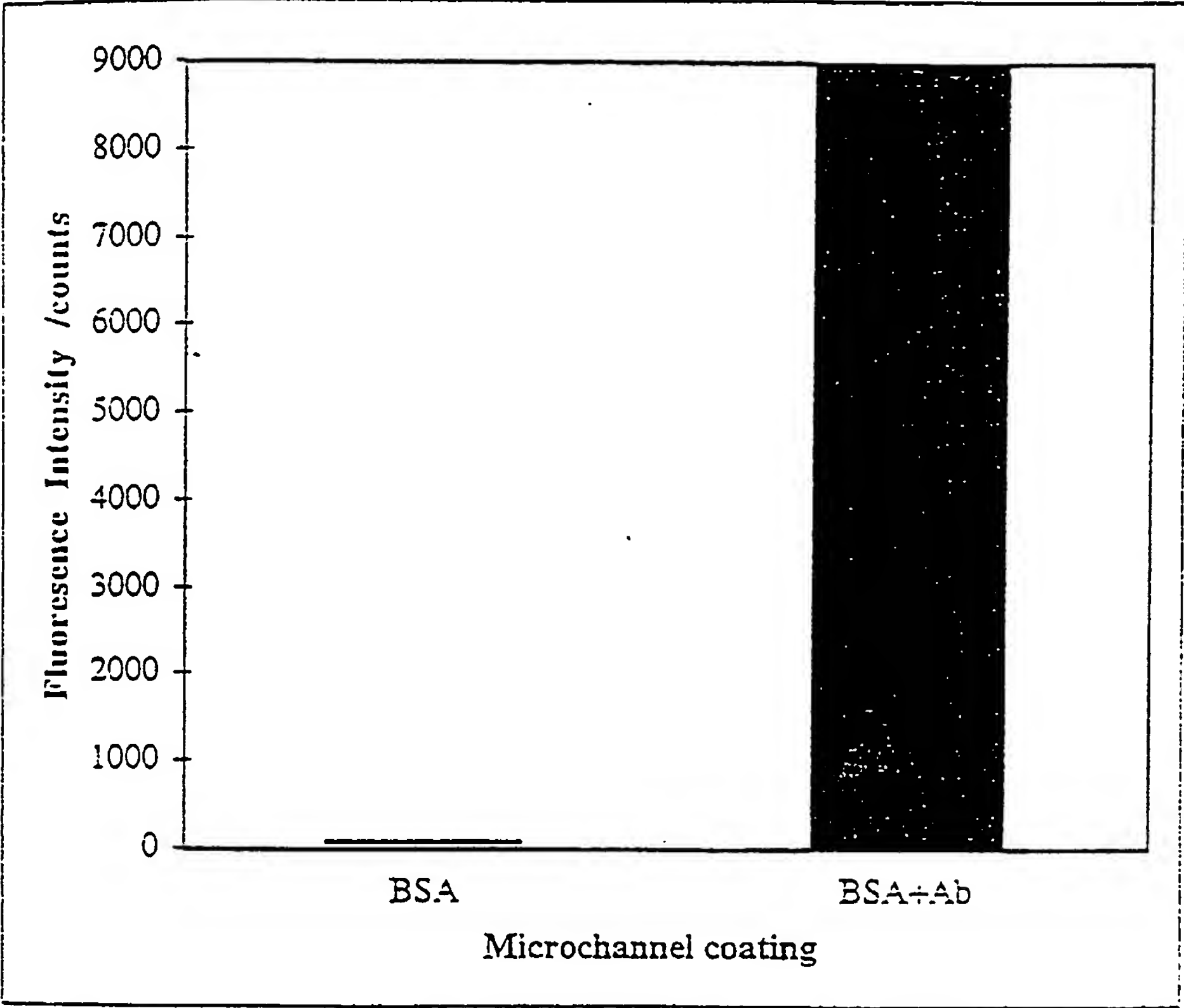
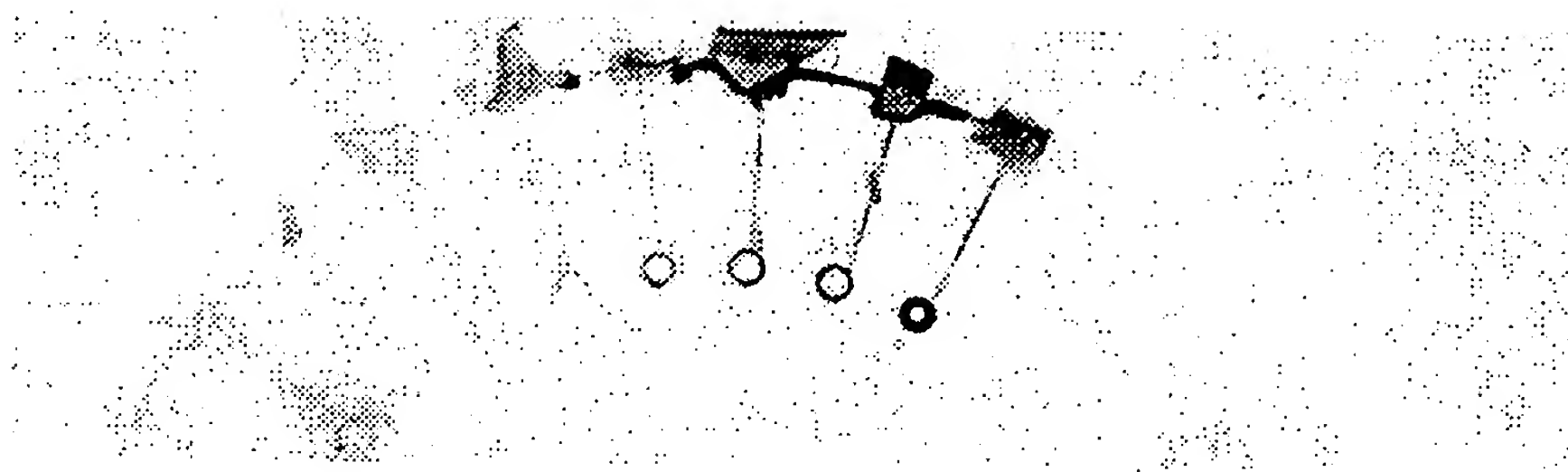


FIG 8

Figure 9:



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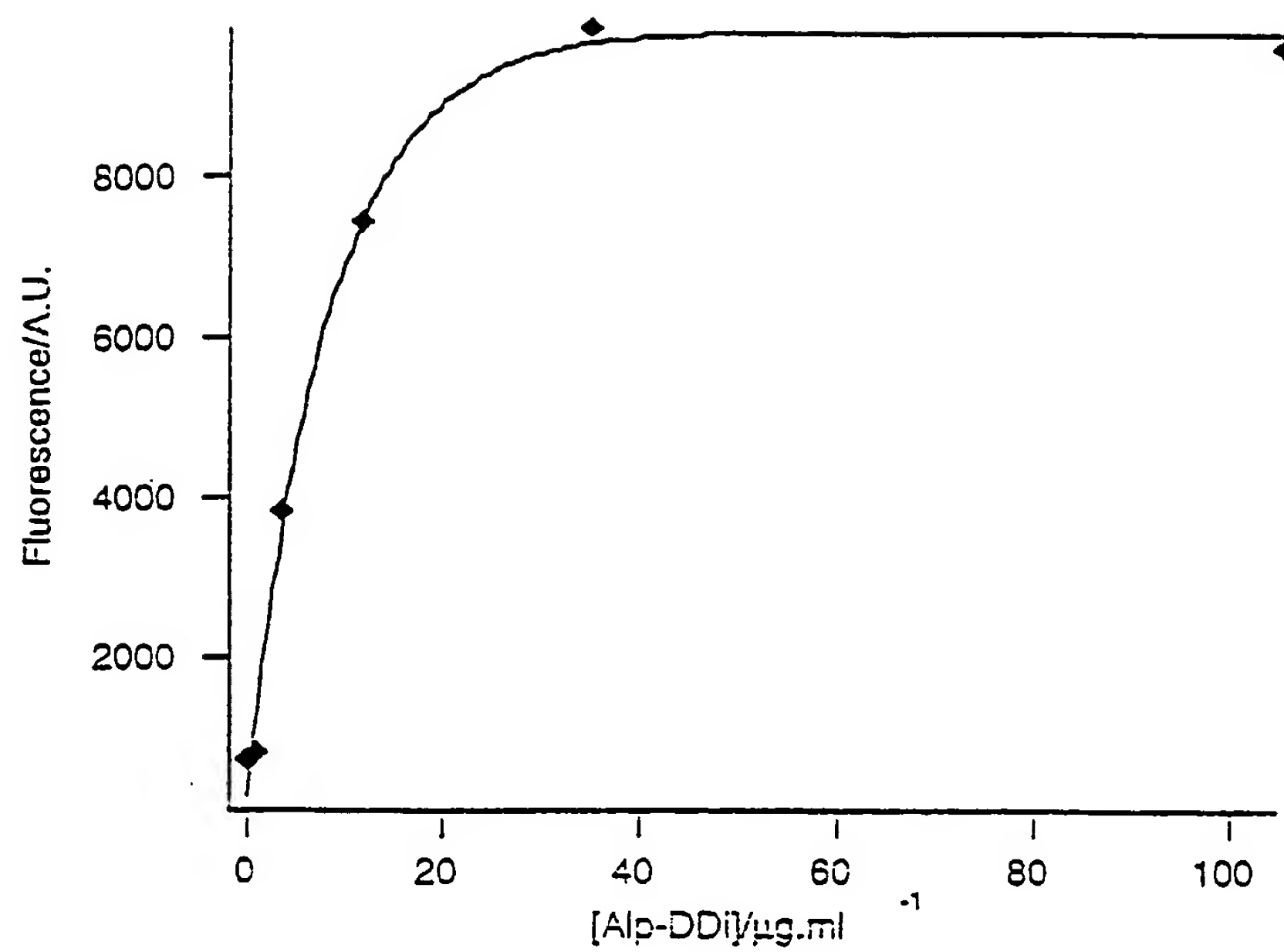


FIG 10

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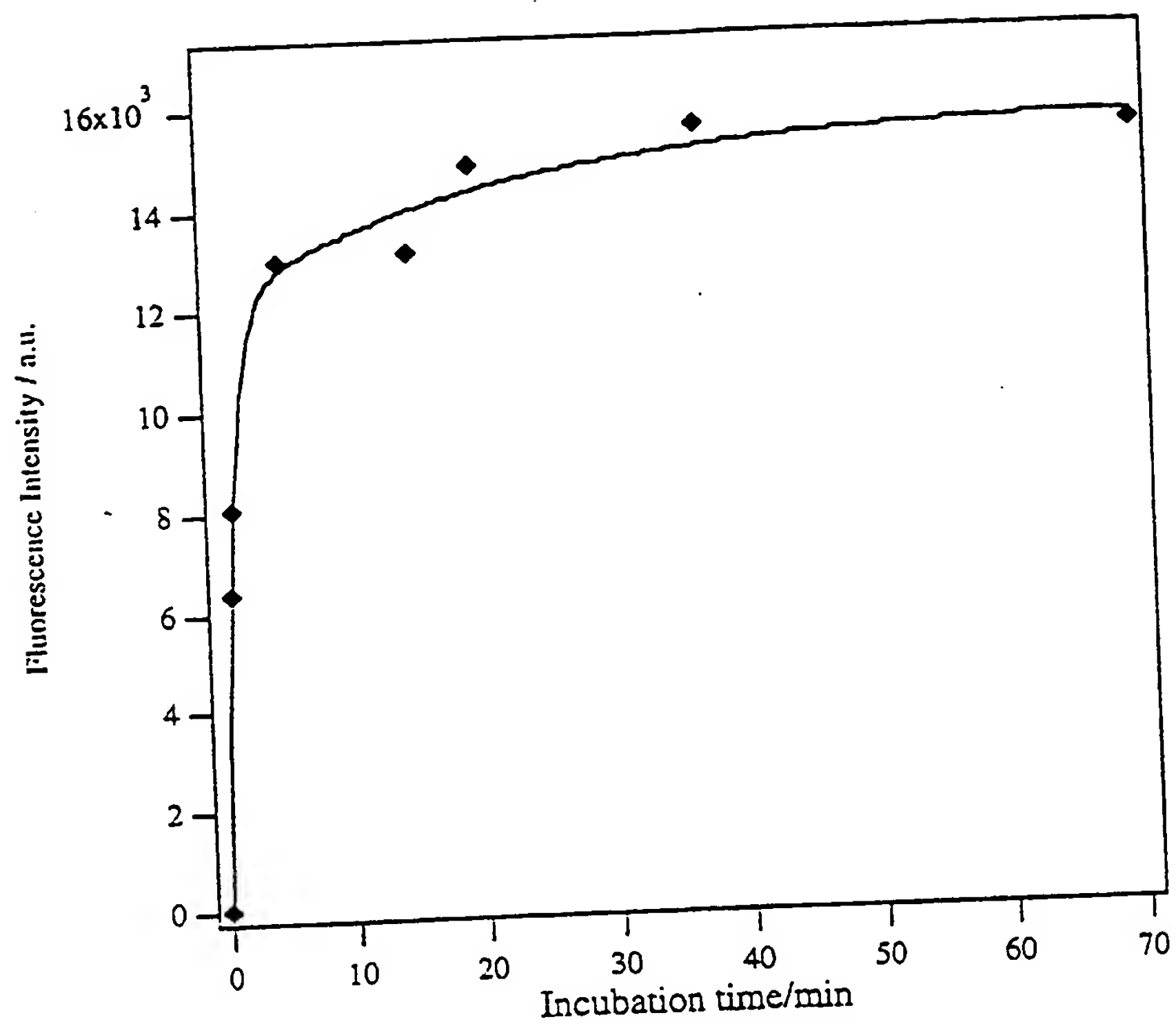


FIG 11

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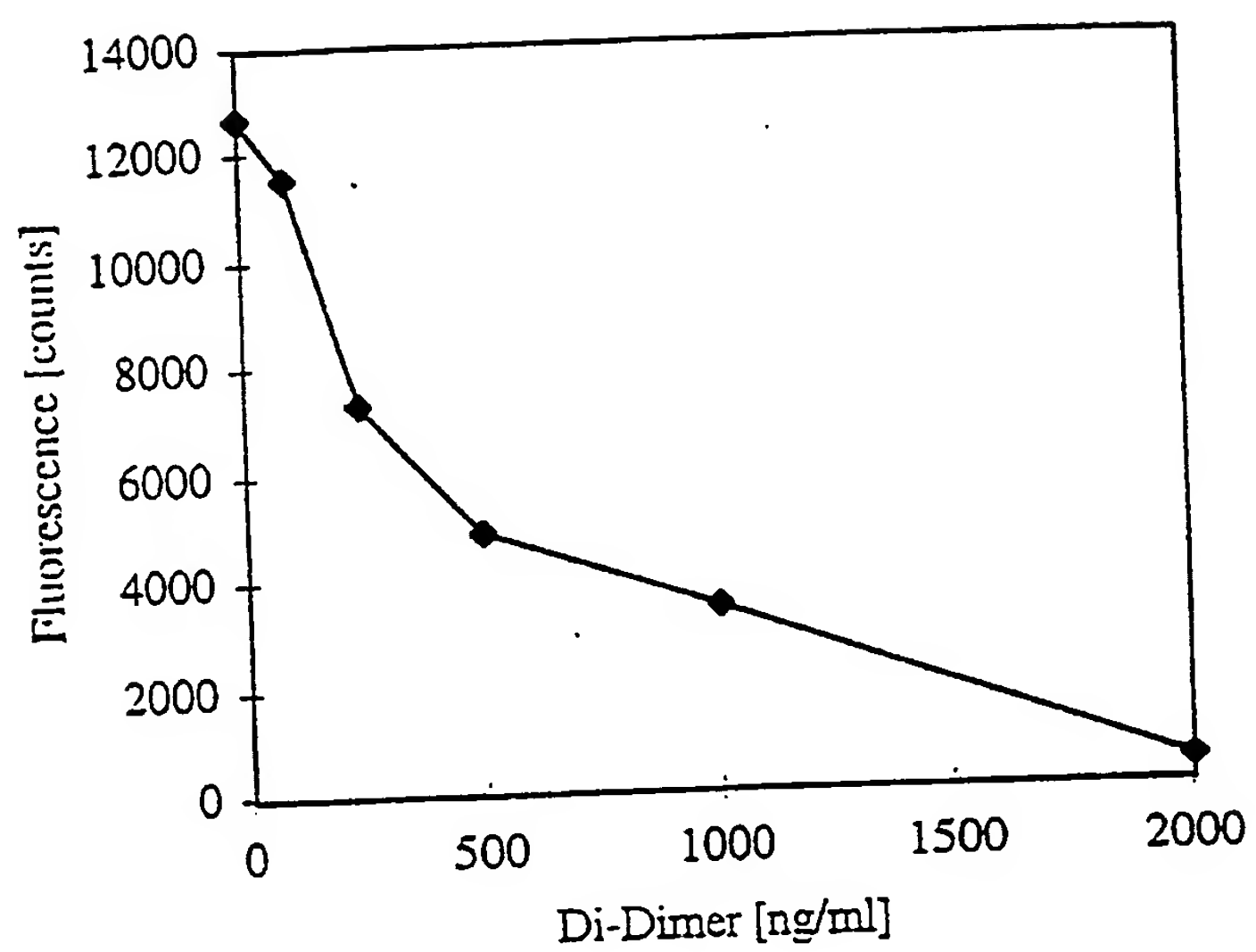


FIG 12

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/02887

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CARLO D. EFFENHAUSER, GERARD J. M. BRUIN, ARAN PAULUS AND MARKUS EHRAT: "Detection of Single DNA Molecules and DNA fragment Analysis in Moulded Silicone Elastomer Microchips" PROCEEDINGS TAS 96, ANALYTICAL METHODS & INSTRUMENTATION, SPECIAL ISSUE UTAS'96, 1996, pages 124-125, XP000916638 cited in the application page 124 -page 125 ---	1-59
X	WO 98 49344 A (LOCKHEED MARTIN ENERGY RESEARC) 5 November 1998 (1998-11-05) claims 33-68 ---	1-59
Y	EP 0 649 534 A (ABBOTT LAB) 26 April 1995 (1995-04-26) claims; figures ---	1-59
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

28 July 2000

Date of mailing of the international search report

17/08/2000

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GONCALVES M L F C

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/EP 00/02887

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 23773 A (SHIPMAN GREGORY K ;UNIVERSAL HEALTHWATCH INC (US); BERNSTEIN DAVID) 3 July 1997 (1997-07-03) claims; figures ----	1-59
A	TJERKSTRA R W ET AL: "ELECTROCHEMICAL FABRICATION OF MULTI WALLED MICRO CHANNELS" MICRO TOTAL ANALYSIS SYSTEMS. PROCEEDINGS OF THE UTAS WORKSHOP, 13 October 1998 (1998-10-13), pages 133-136-136, XP000874456 summary ----	1-59
A	US 5 017 473 A (WAGNER DANIEL B) 21 May 1991 (1991-05-21) cited in the application claims ----	1-59
A	US 4 621 059 A (ROKUGAWA KYUJI) 4 November 1986 (1986-11-04) cited in the application claims ----	1-59
A	US 5 624 850 A (KUMAR AMIT ET AL) 29 April 1997 (1997-04-29) cited in the application claims ----	
A	US 5 585 069 A (ZANZUCCHI PETER J ET AL) 17 December 1996 (1996-12-17) cited in the application claims -----	1-59

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/EP 00/02887

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